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PRINCIPAL INVESTIGATOR: Arthur A. Hurwitz, Ph.D.

CONTRACTING ORGANIZATION: University of California
Berkeley, California 9472

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Arthur A. Hurwitz, Ph.D.

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Berkeley, California 9472

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One of the major goals of tumor immunotherapy is the induction of tumor-specific T cell responses that will be effective at eradicating disseminated tumors. The studies described in this update take advantage of our current knowledge of T cell activation and inhibitory signals. Using a transplantable mammary carcinoma, we demonstrated that blockade of inhibitory signals mediated by CTLA-4 using a monoclonal antibody synergizes with a cell-based vaccine to promote regression of the unmanipulated tumor in a T cell-dependent mechanism. Our recent studies have taken the information gained using this model and applied it to two models of primary carcinogenesis. Using an N-methyl-N-nitrosourea-induced model of mammary carcinogenesis, we have observed that active rejection of a genetically-modified tumor line induces protection against tumor development whereas vaccination in combination with anti-CTLA-4 did not confer similar immunity. In addition, using a transgenic model of prostate cancer, we have demonstrated that vaccination with a genetically-modified tumor line in combination with anti-CTLA-4 reduces tumor incidence, prolongs survival, and induces a strong intra-prostatic inflammatory response. These results are discussed in the context of the goals of the proposal and additional experiments to be performed in the final year are described.

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Table of Contents:

Introduction:

One of the major goals of tumor immunotherapy is the induction of tumor-specific T cell responses that will be effective at eradicating disseminated tumors. In the past decade, insights into the mechanism of T cell activation have provided a basis for developing immunotherapeutic approaches to treating cancer. It is well established that T cells require two distinct signals to be efficiently activated. The first signal results from the antigen-specific interactions between the T cell receptor (TcR) with antigen-major histocompatibility complex (MHC) complexes on an antigen presenting cell (APC). The second, costimulatory signal involves the antigen-independent interaction of CD28 with B7 on the APC surface (reviewed in (1)). Recently, it has become apparent that costimulation is more complex and involves competing stimulatory and inhibitory signals. The latter are mediated by a second T cell counterreceptor for B7, the CD28 homologue CTLA-4 (2). We and others have shown using both *in vitro* and *in vivo* systems that anti-CTLA-4 antibodies can enhance T cell responses by blockade of inhibitory signals (3-6) and using *in vitro* costimulation assays, that cross-linking CTLA-4 delivers an inhibitory signal (7, 8). In addition, *ctla-4* null mutant mice exhibit a severe lymphoproliferative disorder (9, 10). Together, these findings support the idea that CTLA-4 delivers an inhibitory signal to T cell activation.

The fact that B7 expression is limited to "professional" APC led to the idea that the poor immunogenicity of tumor cells might be due to their failure to express B7. This hypothesis was supported by the demonstration that conferral of B7 expression to tumors can induce potent $CD8^+$ T cell responses that are effective in inducing immunity to a variety of tumors (11). Effective induction of anti-tumor T cell responses have been achieved in other systems that also ultimately rely on costimulation, most notably those involving irradiated tumor cell vaccines that express cytokines (12). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine that can activate professional APC's by upregulating MHC and B7 expression as well as promote growth and activation of dendritic cells. One study demonstrated that expression of GM-CSF by a poorly immunogenic melanoma was the most effective vaccination regimen when compared to 10 cytokines and adhesion proteins (13). It was further shown that vaccination with GM-CSF-expressing tumors recruits host-derived APC for cross-priming of T cells with tumor-derived antigens (14).

Taking advantage of the information learned about the inhibitory role of CTLA-4 in T cell activation, we and others described another approach to enhance anti-tumor immunity (15-17). Using an antibody directed against CTLA-4, we showed that CTLA-4 blockade enhances rejection of B7-transfected tumors, and more strikingly, can induce rejection of unmodified tumor cells. Rejection is also accompanied by immunity to rechallenge.

In certain tumor systems, conferral of B7 expression by gene transfer or administration of anti-CTLA-4 is not sufficient to promote tumor regression. One such tumor is the SM1 mammary carcinoma. It was derived by mutagenesis of a pre-neoplastic mammary epithelial cell line with N-methyl-N-nitrosourea (MNU) and carries a point mutation in codon 12 of the *ras* proto-oncogene. The strength of the SM1 model lies in the ability to use SM1 cells as vaccines for mice mutagenized with MNU following pituitary isografts that, if left untreated, would go on to develop mammary

tumors at a high incidence (~70% at 10 months post-mutagenesis). Our preliminary experiments suggested that transduction of SM1 with both *B7-1* and *ifn-γ* was sufficient to promote regression of SM1 in unmanipulated mice in a T cell-dependent mechanism as well as provide protection against rechallenge with the unmodified SM1 tumor. In addition, rejection of SM1 can provide protection to another syngeneic mammary tumor. This work was recently published (18).

More recently, we have studied the synergy between CTLA-4 blockade and a GM-CSF-expressing tumor vaccine. Our initial findings using the transplantable SM1 tumor line indicated that although neither anti-CTLA-4 nor a GM-CSF-expressing SM1 vaccine (GMSM1) alone were effective at treating SM1 tumors, the combination of both treatments was successful at promoting regression of SM1 tumors. Lymphocyte depletion studies demonstrated that both CD4+ and CD8+ lymphocytes were required for rejection and suggested that the synergy between these two treatments is dependent on host-derived antigen presenting cells priming the anti-tumor response. Concurrently, we performed an initial chemical mutagenesis study that made use of different SM1 variants as tumor vaccines in combination with anti-CTLA-4 treatment. The results from this preliminary study suggested that active rejection of the B7+/IFN-γ-expressing SM1 tumor (γB7SM1) was more effective at producing immunity to tumor formation than vaccination with GMSM1 and anti-CTLA-4.

Body:

Methods:

Our studies on the immunotherapy of tumors have employed two different types of approaches: transplantable, cultured tumors (e.g., SM1) and primary tumors (using mutagenesis or transgenic mice). The SM1 tumor provides a model system for poorly immunogenic tumors as well as mammary tumors. By implanting the SM1 tumor subcutaneously (s.c.) into the shaved backs of mice and giving them an irradiated vaccine on the contralateral side, we can follow the growth of the live, parental tumor and assess effectiveness of the vaccine and/or antibody treatments administered intraperitoneally (i.p.). The SM1 tumor was transduced using an ecotropic retrovirus carrying either the murine *ifn-γ* or *gm-csf* genes and cloned by limiting dilution. The resulting lines were irradiated (12,000 rad) and used as vaccines by s.c. injection 3 times, every 3 days. Anti-CTLA-4 or control hamster Ig were administered 3 times, every 3 days, starting 4 days after vaccination. Mice were depleted of lymphocytes using anti-CD4 (clone GK1.5) and/or anti-CD8 (clone 2.43).

For mutagenesis studies, female BALB/C mice (6-8 weeks old) (naive, immune to a live challenge with γB7SM1, or vaccinated with GMSM1/anti-CTLA-4) were implanted with a syngeneic pituitary isograft under the kidney capsule and 1 week later, given a single i.p. dose of MNU [as described in (19)]. Mice were monitored weekly for mammary tumors by manual palpation. Tumors were excised and examined by routine histopathologic analysis to confirm neoplasia. Some tumors were excised while mice were anesthetized and mice were subsequently treated with a GMSM1 vaccine and/or anti-CTLA-4. Mice were continuously monitored for tumor incidence at the primary tumor site as well as for other mammary or metastatic lesions. A small number of mice (~10%) died due to an indeterminate wasting disease and/or lymphomas.

Results/Discussion:

As mentioned in the introduction, during this funding period, we reported that the SM1 tumor requires co-expression of both B7-1 and IFN-γ to be rejected by syngeneic hosts (18). Rejection was dependent on T cells and conferred protection to both the parental SM1 tumor as well as another syngeneic mammary tumor. These findings suggested that SM1 is a weakly immunogenic tumor. Subsequent vaccination studies confirmed this idea (figure 1). Only about 50% of mice vaccinated with irradiated SM1 or B7-1-expressing SM1 cells were immune to rechallenge with the parental tumor. In contrast, all mice vaccinated with either GMSM1 or SM1 co-expressing IFN-γ and B7 (γB7SM1) were immune. These findings were consistent with the idea that SM1 is inherently weakly immunogenic but its immunogenicity can be enhanced by transduction with genes encoding immunostimulatory cytokines like GM-CSF or IFN-γ.

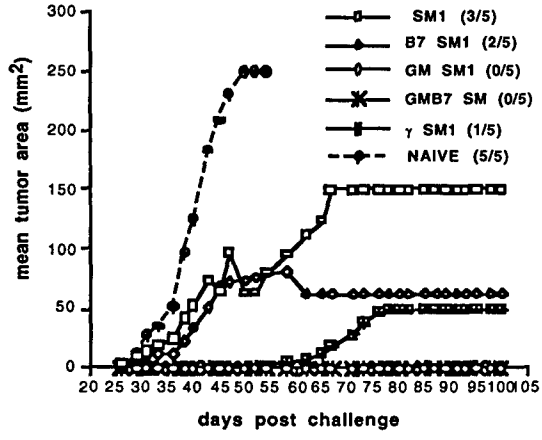

Figure 1: SM1 is a weakly immunogenic tumor. Mice were vaccinated s.c. with 1 x 10^6 irradiated cells of the indicated cell line. 30 days later, mice were rechallenged with 2 x 10^5 live SM1 cells and tumor growth monitored. Incidence of SM1 tumors is indicated in parentheses.

Specific Aim 1:

Our findings presented in the grant proposal demonstrated that neither vaccination with the GM-CSF-expressing vaccine (GMSM1) nor CTLA-4 blockade by administration of an anti-CTLA-4 antibody alone was sufficient to promote regression of SM1. In contrast, mice implanted with an SM1 tumor and treated with the GM-CSF-expressing vaccine followed by anti-CTLA-4 rejected the SM1 tumors(figure 2). Rejection of the SM1 tumor was associated with complete immunity to rechallenge with the parental SM1 tumor. In some studies, where the tumor challenge was reduced to 2x10^4 cells (10-fold greater than the minimum tumorigenic dose of SM1), anti-CTLA-4 treatment alone slowed the growth of the SM1 tumors, but had no significant effect on tumor incidence. Similar to our findings in other studies, we did not observe a synergistic effect between anti-CTLA-4 and a B7-1-expressing SM1 vaccine.

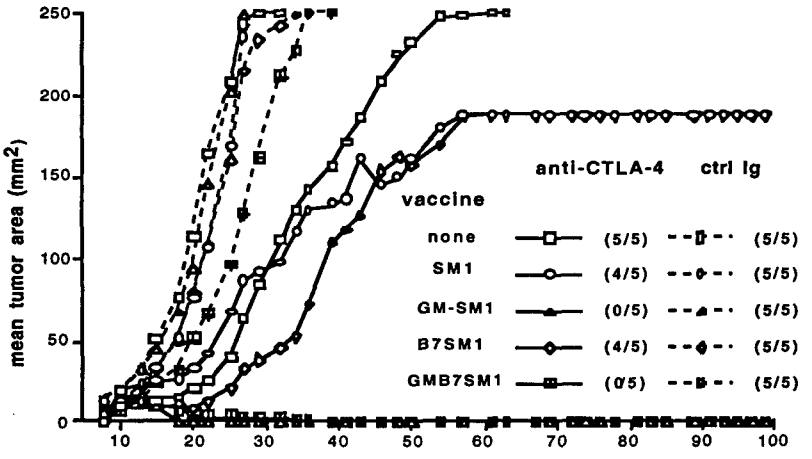

Figure 2: GM-CSF and anti-CTLA-4 synergize in treatment of SM1 tumors. On day 0, mice were implanted with 2×10^4 SM1 cells. On days 0, 3, and 6, mice were injected s.c. on the contralateral flank with the 1×10^6 irradiated cells of the indicated vaccine. On days 4, 7, and 10, mice were injected i.p. with either control antibodies (dashed lines) or anti-CTLA-4 (solid lines). Growth of the parental SM1 tumor was monitored and incidence indicated in parentheses.

To address the first part of Specific Aim 1, mice were depleted of T cells as described in the methods and depletion was confirmed using non-cross-reactive antibodies. As shown in figure 3, the synergism between CTLA-4 blockade and the GMSM1 vaccine was dependent on both CD4+ and CD8+ T cells. Not surprisingly, SM1 tumors grew in mice depleted of both CD4+ and CD8+ cells, despite a treatment regimen that was effective in mice previously administered a control rat IgG suspension. Depletion of CD8+ cells also resulted in tumor outgrowth, consistent with the idea CD8+ CTLs are the effector population mediating anti-tumor cytotoxicity. In addition, SM1 tumors also grew in mice depleted of CD4+ cells alone. Given that SM1 does not express class II MHC, these data imply that GM-CSF expression by the vaccine recruits and activates host-derived APCs that present class II-restricted antigens to CD4+ T cells and that this cross-priming may provide T cell help necessary for elimination of SM1 tumors. Accordingly, CTLA-4 blockade may block inhibitory interactions between these APCs and anti-tumor T cells.

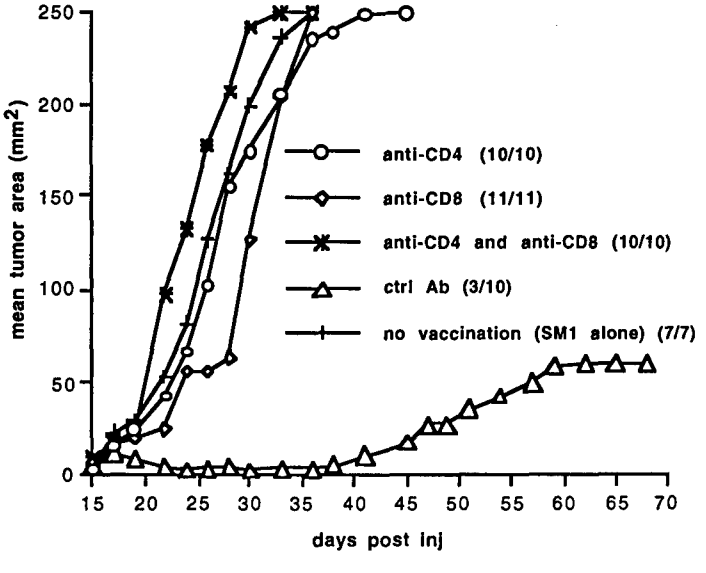

Figure 3. Both CD4+ and CD8+ cell are required for regression of SM1 tumors. 6 days prior to SM1 tumor challenge and initiation of treatment, mice were depleted of the indicated cell population. Mice were implanted with SM1 tumors and treated as described in figure 2. In contrast to mice mock-depleted (triangles) all mice depleted of either CD4+ or CD8+ cells (or both populations) grew tumors.

The second part of Specific Aim 1 was to test the role of costimulation in the rejection of SM1 using CTLA-4 Ig to block B7 interactions with their cognate ligands. . Because our anti-CTLA-4 clone reacts with murine CTLA-4 Ig, we needed to use the chimera consisting of human CTLA-4 extracellular region and the human IgG1 Fc region. In the past few months, our lab has had considerable difficulty preparing sufficient quantities of this fusion protein from the original transfected cell line. However, we recently obtained a newly-derived CHO cell line transfected with the human CTLA-4 Ig construct. Supernatant from this line will be purified over Protein A-Sepharose columns and used to address the role of costimulation by host-derived APCs (as SM1 is B7-negative). Because the first part of Specific Aim 1 is complete [and in press (20)], completion of the CTLA-4 Ig blocking studies will be performed during a similar time frame.

Although the detailed mechanism of rejection in this system remains to be established, our studies demonstrate that both CD4+ and CD8+ cells are required. Because SM1 does not express MHC class II, even after exposure to IFNγ (18), the requirement for CD4+ cells suggests that class II-restricted, CD4+ T cells are primed by host APCs. This idea is consistent with previous reports that tumor-derived GM-CSF enhances host presentation of tumor antigens and permits cross-priming to occur (14). GM-CSF is known to promote growth and activation of dendritic cells and render them more potent APCs (21-23). Accordingly, CTLA-4 blockade in this treatment regimen may block inhibitory interactions between host APCs (potentially GM-CSF-stimulated dendritic cells) and T cells, and facilitate costimulatory interactions between APC and T cells, thereby enhancing priming of T cells to promote immunity to and rejection of SM1.

Our previous findings suggested that both IFNγ and B7 enhanced immunogenicity of SM1 by directly enhancing T cell activation (18). They also suggested that if "co-therapies" were to act synergistically, they both needed to activate the same 'arm' of T cell activation (i.e., enhancement of T cell activation by the tumor as APC or antigen presentation by host-derived APC). Consistent with this idea, the data in the current study demonstrated that CTLA-4 blockade did not synergize with B7 expression by the tumor but it did synergize with tumor-derived GM-CSF expression. Accordingly, both tumor-derived GM-CSF expression and CTLA-4 blockade presumably enhance T cell activation at the level of host-derived APC function and therefore result in successful T cell priming. In contrast, by enhancing two different mechanisms of T cell priming (enhancing APC function of a tumor by conferring B7 expression and enhancing host APC function by CTLA-4 blockade), efficient T cell activation and therefore tumor clearance could not take place.

To address further the mechanism of synergy between anti-CTLA-4 and GM-CSF-expressing vaccines, we are performing histopathological analysis. Biopsies from the vaccination site, tumor and draining lymph nodes are being taken at 6, 12, and 20 days after tumor challenge/vaccination. These studies will include immunolocalization of T cells, B cells, macrophages, dendritic cells, and NK cells as well as histological stains that identify various populations of myeloid cells (macrophages, eosinophils, and neutrophils). By characterizing the infiltrating cells at the vaccination site, we hope to identify cells responsible for antigen recruitment and processing as well as presentation. By identifying cells infiltrating the tumor site, we hope to elucidate the effector cell mechanisms. Finally, examination of the draining lymph nodes will permit elucidation of the priming process. Although not originally described in the proposal, these studies will significantly

contribute to a greater understanding of the mechanism of the synergy between GM-CSF-expressing vaccines and CTLA-4 blockade.

Specific Aim 2:

As mentioned above, a major goal of immunotherapy is the elimination of established tumors. Our previous studies (figure 2) used treatment of SM1 tumors starting at the time of injection. In these studies, s.c. tumors grew to an average of 30 mm^2 before regressing in all of the animals. When the initial tumor challenge was increased 10-fold, the treatment efficiency was significantly reduced and treatment with anti-CTLA-4 alone had no effect on tumor growth (20). To address the goal of Specific Aim 2 (treatment of a more established tumor), we initiated studies wherein mice were implanted with the lower dose of SM1 cells and treatment with the GMSM1 vaccine and anti-CTLA-4 were delayed four days. In these studies, where tumor growth is still not detectable at the time of treatment, even the combined GMSM1/anti-CTLA-4 regimen had no effect on tumor growth (data not shown). These two sets of data (increased initial tumor burden and delayed treatment) suggest that in the SM1 model, the rate of tumor growth exceeds the ability of the immune system to mount an effective response and that treatment at the time of tumor challenge is at the threshold of a window of effectiveness. Alternatively, the rapidly-growing SM1 tumor (that requires euthanasia of all control-treated mice by 3.5 weeks after challenge with 20,000 cells) may induce a state of tolerance in anti-SM1 T cells that cannot be overcome by the GMSM1/anti-CTLA-4 treatment begun only four days after tumor challenge. Similar findings have been observed using a B16 melanoma model where only anti-CTLA-4/GM-CSF-expressing vaccine is effective (Van Elsas, Hurwitz, and Allison, manuscript in preparation).

Specific Aim 3:

As described above, rejection of SM1 tumors as a consequence of CTLA-4 blockade and vaccination with a GM-CSF-expressing vaccine results in immunity to rechallenge with the parental SM1 tumor, which was one component of Specific Aim 3. In our studies using the γB7SM1 tumor, rejection conferred protection to both SM1 and another syngeneic mammary carcinoma, MOD (18). Because protection against MOD challenge was complete following rejection of γB7SM1 and the GMSM1/anti-CTLA-4 treatment regimen was determined to be more effective than treatment with a γB7SM1 vaccine alone (as discussed in the proposal, our preliminary treatment experiments demonstrated that γB7SM1 was not effective at treating growth of the parental SM1 tumor), we decided that additional studies of cross-protection would be redundant. Therefore we have focused our efforts on determining the requirements for immunotherapy of primary tumors using the MNU mutagenesis model (Specific Aim 4) and a prostate cancer model (see below).

Specific Aim 4:

One of the strengths of using the transplantable SM1 mammary tumor line is that it can be used as a vaccine for syngeneic mice that develop mammary tumors after induced as a consequence of chemical mutagenesis (19). In this system, mice are implanted with a pituitary isograft to induce proliferation of the mammary tissue and subsequently mutagenized with MNU (50 mg/kg).

Although this protocol differs slightly from the one in the research proposal, our collaborators in the laboratory of Dr. Satyabrata Nandi have determined this to be more efficient at mammary tumor induction. We therefore proposed to use this mutagenesis system to test the ability of SM1-based vaccines to confer protection to mammary carcinogenesis. In addition, we have attempted to treat mice that had their primary mammary tumors extirpated using similar vaccination approaches.

We recently concluded our first carcinogenesis experiment that (to our surprise) lasted 11 months. Naive mice, mice that had previously rejected γB7SM1, and mice treated with a GMSM1 vaccine were mutagenized as described above and monitored for mammary tumor development. Mammary hyperplasia was detected as enlarged nodules by 3 months after mutagenesis whereas frank tumors were not first detectable until 5 months after MNU treatment. However, the majority of mammary tumors did not occur until 6-8 months after mutagenesis.

One week after resection of primary tumors, groups of 10 (previously naive) animals were treated with a GMSM1 vaccine and anti-CTLA-4. In this group (as well as sham-treated control mice), mammary tumors arose at both the original site (10/10 in vaccinated mice) as well as in additional mammary glands that necessitated euthanasia of the mice. These data indicated that the SM1 vaccines were not capable of treating previously established mammary tumors where minimal residual disease existed.

To assess 'protection' of mice against mammary tumorigenesis, mice that rejected the γB7SM1 tumor or mice treated with GMSM1 vaccine and anti-CTLA-4 were subsequently mutagenized as described above. Tumor incidence in unmanipulated mice was 72% (33/46) whereas incidence in γB7SM1-primed mice was only 33% (3/9, P=.05, Fisher's Exact Test). In contrast, mammary tumor incidence in mice treated with the irradiated GM-CSF-expressing vaccine and anti-CTLA-4 was 86% (6/7). Interestingly, the GMSM1 vaccine induced immunity to all mice rechallenged with the parental SM1 tumor (figure 1, above) whereas rejection of the γB7SM1 only protected 75% of mice to SM1 rechallenge (18). In contrast, these mutagenesis data suggest that 'active' immunization is more effective than 'passive' vaccination at preventing mammary tumor formation in this particular model. However, the small cohort number of each group necessitates cautious analysis of the data and larger group sizes in future experiments.

Given the generally poor treatment efficiency in this initial experiment, several modifications were made for the next experiment. First, each group will consist of 25 mice. Second, we established a new mammary tumor line from a mouse that developed a metastatic tumor as a consequence of MNU treatment. This line, "1301", is tumorigenic in BALB/C mice and was transduced to express GM-CSF for use as a vaccine. Third, mice for "treatment" groups will be treated 3-4 months after MNU injection rather than after tumor extirpation. In addition, given the high incidence of codon 12 *ras* mutations in MNU-treated mice (19), a group of mice will be vaccinated with a mutant ras peptide known to induce a proliferative response in BALB/C mice (24). Finally, emphasis will be put on studying the "protection" groups to determine whether active rejection of the SM1 tumor does provide more potent immunity than passive vaccination with the SM1 lines.

In terms of the Statement of Work for Specific Aim 4, several points will be changed. First, given the length of each individual study (and the resulting animal cage costs), emphasis will be placed on making one more interpretable study during the funding period. Second, lymphocyte

depletion studies will not be performed due to the difficulty of maintaining depletions for such an extended period. However, future studies may address this issue by studying the role of T cell subsets in the initial priming phase. Finally, treatments will not be staggered as proposed in the last part of Specific Aim 4. Hopefully, these modifications will still permit us to use this interesting model of mammary carcinogenesis and yield more interpretable results.

More recently, our studies have also focused on immunotherapy of prostatic disease using a transgenic model of prostate cancer (TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP mice) (25). In this model, TRAMP mice express the SV40 large T antigen (TAg) under the transcriptional control of the probasin promoter. By 12 weeks of age, male TRAMP mice exhibit histological signs of disease discernible as prostatic intraepithelial neoplasia (PIN) and cribiform structures. Adenocarcinoma can be detected at 18 weeks of age, and by 28 weeks, 100% of the male TRAMP animals will have advanced disease, many exhibiting metastatic disease (26). We previously demonstrated that two cells lines derived from TRAMP mice (TRAMPC1 and TRAMPC2) were rejected by non-transgenic C57BL/6 mice following administration of anti-CTLA-4 or transduction of the cells to express B7-1 (16). Using the information learned from the SM1 mammary tumor system on the synergy between GM-CSF vaccines and CTLA-4 blockade, a cohort of 180 TRAMP mice were subdivided into 6 groups. At 16 weeks of age, mice were vaccinated s.c. with an irradiated cell vaccine (TRAMPC or GM-CSF-expressing TRAMPC cells [GM-TRAMPC]) and treated i.p. with anti-CTLA-4 or a control antibody. Three weeks after treatment, 4 animals per group were euthanized, tissues were fixed and processed for histopathological analysis and the following criteria assessed: tumor incidence, urogenital tract weight, prostate weight, and metastasis. At this early time point, the most striking finding was the low tumor incidence in mice treated with anti-CTLA-4, irrespective of vaccination (3/12 vs 11/12). Therefore, the remaining 25 mice per group were euthanized 5 weeks later and similar analyses performed. At this later time point, there was a significant reduction in tumor incidence among mice treated with anti-CTLA-4 and vaccinated with either TRAMPC or GM-TRAMPC ($p=.05$ and $p=.009$, respectively, figure 4). In addition, histopathologic analyses to date have revealed a less severe histologic progression of prostatic disease (figure 5). Most notably, there was marked accumulation of mononuclear cells in the inderductal spaces of the dorsal and lateral prostate lobes of mice vaccinated with GM-TRAMPC and treated with anti-CTLA-4. In addition, parallel studies performed by our collaborators at the NIH demonstrated significantly enhanced survival of TRAMP mice undergoing similar treatment regimens; at 9 months of age, survival is 75% in vaccinated mice compared to 10% in control-treated animals. Additionally, these findings support the idea that transplantable TRAMPC lines serve as potent vaccines, despite their lack of TAg expression.

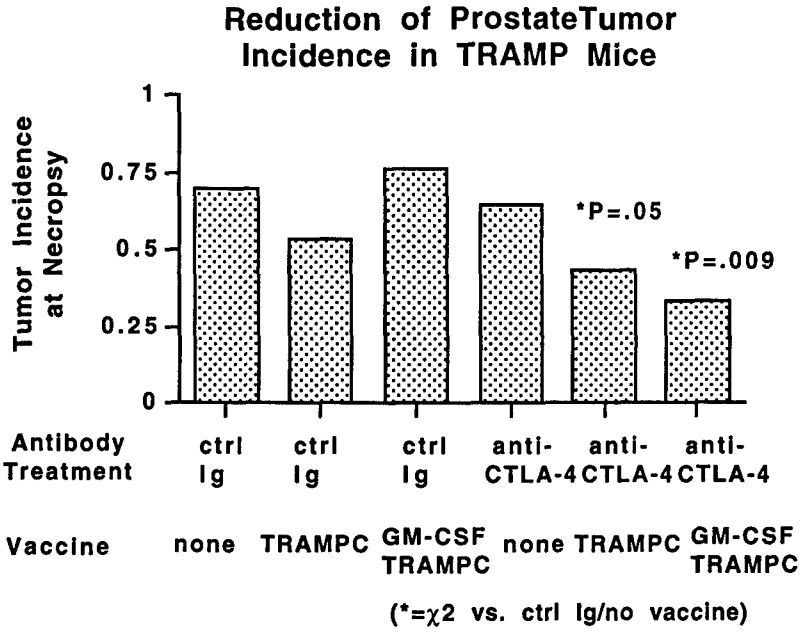

Figure 4. TRAMP mice were treated with the indicated vaccine and antibody at 16 weeks of age. 8 weeks later, mice were euthanized and tumor incidence assessed at necropsy. Tumor incidence was confirmed by histopathology.

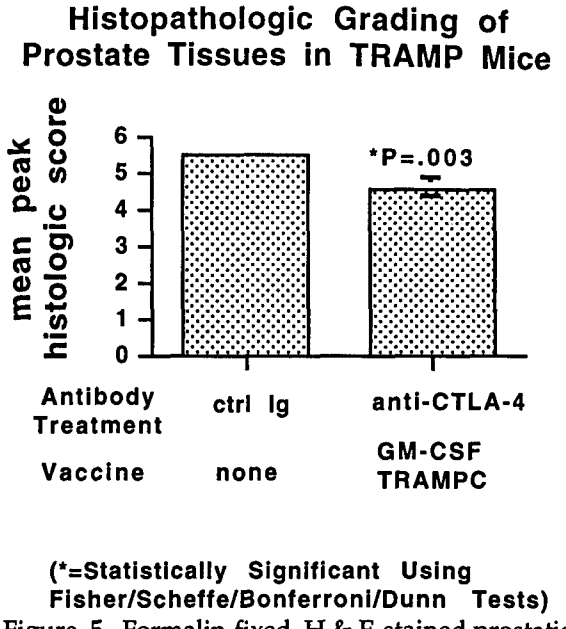

Figure 5. Formalin-fixed, H & E-stained prostatic tissues were scored blindly according to the following scale: 1=normal; 2=low-grade PIN; 3=high grade PIN/cribiform structures; 4=loss of interductal spaces; 5=invasive adenocarcinoma with loss of lumenal spaces; 6=sheets of undifferentiated tumor.

Conclusions:

The ultimate goal of tumor immunotherapy is the induction of tumor-specific T cell responses capable of eradicating tumor cells and preventing recurrent disease. This has proven to be difficult because despite expression of antigens that are recognizable by the host immune system, tumor cells are very poor at initiating effective T cell responses. However, elucidation of the regulation of T cell activation has contributed significantly to developing immunotherapeutic approaches to treating cancer.

Our findings using the transplantable SM1 tumor demonstrating the synergy between GM-CSF and CTLA-4 blockade support the idea that breast cancer may be a suitable candidate for immunotherapeutic intervention(20). In addition, the cross-protection studies using syngeneic mammary tumors (18) suggest that tumor antigens may be shared and taken together with other studies (27, 28), suggest that a protective vaccine may be feasible. Our preliminary results using the MNU carcinogenesis model are somewhat encouraging and merit further study. As described above, significant reduction in tumor incidence was observed in mice that had previously rejected the γB7SM1 tumor. Future studies will incorporate larger cohorts and another recently developed mammary tumor line for use as a vaccine. Mice that do appear to have tumor immunity will be tested for T cell function using a panel of BALB/C-derived mammary tumors as well as a mutant ras peptide. Finally, our findings from separate studies using a transgenic model of prostate cancer further support the use of immunotherapy for the treatment of primary tumors.

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ENHANCEMENT OF THE ANTI-TUMOR IMMUNE RESPONSE USING A COMBINATION OF INTERFERON-γ AND B7 EXPRESSION IN AN EXPERIMENTAL MAMMARY CARCINOMA

Arthur A. HURWITZ[1,2]*, Sarah E. TOWNSEND[2,3], Tina F.-Y. YU[2], Jeanette A. WALLIN[2] and James P. ALLISON[1,2]

[1]*Howard Hughes Medical Institute University of California, Berkeley, CA, USA*
[2]*Cancer Research Laboratory, University of California, Berkeley, CA, USA*
[3]*Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA, USA*

In recent years, tumor immunotherapy has begun to exploit the emerging knowledge of the mechanisms of T cell activation to enhance the immune responses to tumors. However, many tumors, despite genetic modification to express co-stimulatory molecules or cytokines, are not readily rejected due to their inherently poor immunogenicity. In the present study, we tested whether expression of the co-stimulatory ligand B7-1 and the immunostimulatory cytokines interferon gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) by a mammary carcinoma (SM1) would sufficiently augment its immunogenicity to obtain rejection and immunity. Our findings demonstrate that expression of B7, IFN-γ, or GM-CSF alone, or co-expression of B7 and GM-CSF did not result in rejection of SM1. However, co-expression of B7 and IFN-γ was sufficient to result in regression of SM1 tumors by a CD8$^+$ T cell-dependent mechanism. Rejection of the B7/IFN-γ-expressing SM1 tumor resulted in protection from rechallenge not only with the unmodified SM1 tumor but with another syngeneic mammary tumor. Our data support the idea that although B7 expression alone may not be sufficient for rejection of certain tumors, the immune system may be stimulated to mount an effective anti-tumor immune response by the co-expression of both the co-stimulatory ligand and a cytokine. *Int. J. Cancer* 77:107–113, 1998.
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Initiation of an effective T cell-mediated immune response requires 3 principle components: (1) a particular antigen capable of being recognized by the T cell antigen receptor (TcR), (2) expression of major histocompatibility complex (MHC) antigens by an antigen presenting cell (APC) that presents the particular antigen associated with MHC on its surface, and (3) co-stimulatory signals or help provided by an exogenous source of growth factors. A T cell-mediated immune response to tumors requires that these same criteria also be met (reviewed in Allison *et al.*, 1995). A tumor may escape immune surveillance due to the lack of expression of any or all of these components. As a result, these tumors may become non-immunogenic.

Several approaches have been taken to enhance the immunogenicity of tumors. Many studies focused on conferring cytokine expression to tumors by gene transfer techniques. By providing cytokine expression to tumors, autocrine effects on MHC expression or paracrine effects on APC or T cells may sufficiently boost the immune response to promote tumor immunity. Interleukin-2 (IL-2) (Fearon *et al.*, 1990) and IL-4 (Golumbek *et al.*, 1991), both important in providing help during T cell activation, were demonstrated to augment T cell immunity to tumors of various tissue origins. Interferon-gamma (IFN-γ), a T cell-derived cytokine that can modulate T cell activation by either directly activating the effector T cell or upregulating MHC expression on APCs was also capable of enhancing tumor immunity (Gansbacher *et al.*, 1990) in a T cell-dependent mechanism. In addition to promoting the growth and differentiation of myeloid cells, granulocyte-macrophage colony-stimulating factor (GM-CSF) has pleiotropic effects that can result in APC activation by modulating MHC and co-stimulatory molecule expression (Morrissey *et al.*, 1987). Immunization with irradiated, GM-CSF-expressing melanoma cells was the most effective regimen compared to 10 cytokines and adhesion proteins (Dranoff *et al.*, 1993); protection was dependent on both T cells and NK cells (Levitsky *et al.*, 1994). The demonstration that GM-CSF was the most effective cytokine of those tested suggests recruitment of professional APCs could enhance the potency of tumor cell vaccines. However, an alternative approach to this idea would be to endow the tumor cells themselves with greater APC function.

In addition to antigen-specific signals through the TcR, an antigen-independent co-stimulatory signal is required for efficient T cell activation. A co-stimulatory signal through CD28 in combination with TcR triggering result in T cell activation as measured by cytokine expression and proliferation (reviewed in Allison, 1994). Two co-stimulatory ligands for CD28 have been identified: B7-1 and B7-2 (Freeman *et al.*, 1993; Linsley *et al.*, 1994). Both are expressed by "professional" APCs such as activated B cells, monocytes, and dendritic cells. However, B7 expression is principally restricted to cells of the hematopoietic lineage. Therefore, tumors not of hematopoietic origin may not constitutively express either member of the B7 family or they down-regulate B7 expression, thereby providing a mechanism to avoid activation of T cells (Denfeld *et al.*, 1995).

Using gene transfer techniques, we and others demonstrated that conferring B7-1 expression to tumors of a variety of tissue origins was, in many cases, sufficient to promote rejection (Chen *et al.*, 1992; Townsend and Allison, 1993). These studies demonstrated that tumors of both lymphoid and non-hematopoietic origins transduced to express B7-1 were rejected by a CD8$^+$ T cell-dependent mechanism and that B7-mediated tumor rejection correlated with tumor immunogenicity (Chen *et al.*, 1994). It has been suggested that NK cells could also be recruited to eliminate B7$^+$ tumors (Wu *et al.*, 1995). However, in certain tumor systems, B7 expression was not sufficient to promote rejection. This could be attributed to low level or loss of expression of certain MHC loci and/or tumor antigens, lack of adhesion molecule expression, secretion of immunosuppressive cytokines, or other mechanisms that decrease tumor immunogenicity (Chen *et al.*, 1994). In 2 studies, this problem was overcome by conferring both B7-1 and IL-12 expression to the tumor; rejection was dependent on T cells as well as IFN-γ expression (Coughlin *et al.*, 1995; Zitvogel *et al.*, 1996).

In the present study, we describe the requirements for T cell-mediated rejection of an *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinoma, SM1. Whereas neither B7-1, GM-CSF, nor IFN-γ expression alone nor co-expression of B7-1 and GM-CSF were sufficient to promote regression of SM1, co-expression of B7-1 and IFN-γ resulted in (CD8$^+$) T cell-dependent

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*Correspondence to: Cancer Research Laboratory, 415 Life Sciences Addition, University of California, Berkeley, CA 94720, USA. Fax:(510)642-5741. E-mail: hurwitz@socrates.berkeley.edu

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tumor rejection. Mice that rejected γB7SM1 were protected against subsequent challenge with the unmanipulated (parental) SM1 tumor as well as another BALB/c-derived mammary tumor. These data support the idea that although B7 expression alone may not be sufficient for rejection of certain tumors, the immune system may be modulated to mount an anti-tumor immune response to a poorly immunogenic tumor by the co-expression of both a T cell co-stimulatory ligand as well as a cytokine.

MATERIAL AND METHODS

Cell lines

The BALB/c-derived mammary carcinoma SM1 was derived in the laboratory of Dr. Satyabrata Nandi (University of California, Berkeley) (Guzman *et al.*, 1992). Briefly, a pre-neoplastic, BALB/c-derived mammary cell line was mutagenized with MNU *in vitro* and injected into the cleared fat pad of syngeneic mice. Palpable tumors from the mammary tissue were excised and placed into tissue culture after enzymatic release from extracellular matrices. Tumor lines serially passaged through syngeneic mice were selected for malignant phenotype. SM1 was selected as a line that consistently caused tumors at low inocula (tumorigenic at or below 5,000 cells) and stained positive for vimentin protein expression by immunohistochemistry. It was then cultured in MEM Eagle's (UCSF Cell Culture Facility, San Francisco, CA) supplemented with 10% FCS, 1 X MEM non-essential amino acids, L-glutamine, and MEM vitamins (Biowhittaker, Walkersville, MD). Cells were lifted from tissue culture dishes using either 5 mM EDTA in calcium/magnesium-free saline or 0.25% trypsin solution (Biowhittaker). MOD and EL-12 were obtained as a generous gift from Dr. D. Medina (Baylor College of Medicine, Houston, TX) and grown in DMEM:F12 (Biowhittaker) supplemented with 2% bovine serum (UCSF Cell Culture Facility), 10 μg/ml insulin (Sigma, St. Louis, MO), and 5 ng/ml epidermal growth factor (GIBCO, Gaithersburg, MD). Tumor incidence of MOD and EL-12 was 100% at the dose used (2 X 10^5 cells).

B7-1-expressing lines were prepared as previously described (Townsend and Allison, 1993) using an electroporation method. Briefly, cells in logarithmic growth phase were released from tissue culture dishes by trypsinization and washed in electroporation buffer (250 mM sucrose, 1 mM magnesium chloride in 2 mM PBS, pH 7.4). DNA (100 μg) was added and 10^7 cells were electroplated using electrodes with a 2 mm gap and a setting of 5 pulses of 99 μsec at 550 V. Cells were cultured in selective medium (0.5 mg/ml G418 [GIBCO]) added 48 hr after electroporation. Clones were produced by limiting dilution and screened for B7-1 (hereafter referred to as B7) expression by flow cytometric analysis.

To obtain IFN-γ- or GM-CSF-expressing lines, cells were infected with a retrovirus containing the mouse *IFN-γ* or *GM-CSF* genes driven by the Maloney murine leukemia virus LTR, using the ψCRIP producer line. Retrovirus-containing supernatants were added to SM1 cultures overnight in the presence of 8 μg/ml polybrene (Sigma). Clones were generated by limiting dilution and supernatants tested for cytokine expression by enzyme-linked immunosorbent assay (ELISA) (IFN-γ-Genzyme, Cambridge, MA; GM-CSF-PharMingen, San Diego, CA).

Flow cytometric analysis

Cells were examined for surface expression of B7 and MHC antigens using CTLA-4 Ig or antibodies, respectively, followed by flow cytometric analysis. To quantitate B7 expression, non-specific Ig binding was blocked by incubation of cells with a combination of rabbit Ig and anti-FcγRII (clone 2.4G2, ATCC, Rockville, MD) and then incubated with CTLA-4 Ig (a chimeric molecule consisting of the extracellular domain of CTLA-4 and the Fc domain of human IgG_1) followed by a FITC-conjugated goat anti-human antibody (Caltag, South San Francisco, CA). MHC expression was assessed using hybridoma supernatants containing anti-class I (M1.42.3.9.8.HLK) or class II (N22) monoclonal antibodies (ATCC) followed by the appropriate fluorochrome-coupled, species-specific antibodies (Caltag). Cell suspensions were analyzed on a Coulter EPICS-XL (Hialeah, FL).

Tumor injections

All animal procedure were performed according NIH guidelines under protocols approved by the University of California Animal Care and Use Committee. SM1 cells propagated in culture were harvested with trypsin (Biowhittaker), washed 3 times in balanced salt solution, and resuspended in saline at the indicated concentration. Mice were injected sub-cutaneously (s.c.) into a shaved area on the back with 100 μl of tumor cell suspension. Tumor growth was monitored by measuring bisecting diameters using a caliper. When the tumor area exceeded 250 mm^2, mice were euthanized and a value of 250 mm^2 was entered for each euthanized mouse. This value was used to calculate the mean tumor area until all mice from a given group were euthanized.

To confirm *in vivo* expression of the transduced gene, tumors were excised from mice that had detectable tumors. Tumors were minced using a razor blade and digested using a combination of trypsin and collagenase (Worthington, Freehold, NJ). Resulting cell suspensions were either examined by flow cytometry for B7 expression or cultured and supernatants tested at intervals for cytokine expression as described above.

Antibody treatment in vivo

To neutralize IFN-γ expression, mice were injected i.p. with 0.5 mg or 1 mg of either protein G-purified anti-IFN-γ (clone R46A2, ATCC) or a control rat IgG (Sigma) starting on the day of tumor challenge and continuing every 5 days for the duration of the experiment.

For lymphocyte depletion experiments, mice were injected with anti-CD4 (GK 1.5, 400 μg), anti-CD8 (2.43, 600 μg), a combination of both anti-CD4 and anti-CD8, or control antibody (purified rat IgG, 600 μg, Sigma) 3 times prior to tumor injection (days -6, -5, and -4) as well as once every 10 days subsequent to tumor inoculation. Lymphocyte depletion was confirmed using non-cross-reactive antibodies (CD4: clone CTCD4, Caltag; CD8: CT-CD8β, Caltag) prior to tumor injection by testing peripheral blood or lymph node cells (from control animals) for the appropriate lymphocyte populations.

RESULTS

Using a variety of transplantable tumor lines, our laboratory and others have demonstrated that B7 expression may be sufficient to promote tumor rejection and provide immunity to subsequent tumor challenge. Immunization with $B7^+$ tumor vaccines was also shown to result in rejection of recently established tumors (Baskar *et al.*, 1995), thereby providing a basis for immunotherapeutic protocols. In the present study, we tested the requirements for rejection of a weakly immunogenic mammary carcinoma line. The SM1 tumor is a BALB/c-derived mammary carcinoma resulting from *in vitro* mutagenesis with MNU. We genetically modified SM1 to express the mouse *B7-1* gene as well as IFN-γ or GM-CSF and tested the tumorigenicity of these transduced tumors.

Expression of B7, IFN-γ, or GM-CSF alone is not sufficient to promote tumor rejection

$B7\text{-}1^+$ lines were generated by electroporation of SM1 cells with the murine gene as described previously (Townsend and Allison, 1993). A clone (B7SM1) was selected for injection based on high B7-1 expression and MHC class I expression comparable to the untransfected (parental) tumor (Fig. 1). The level of B7-1 expressed by B7SM1 was comparable to that of the EL4 thymoma and K1735 melanoma that were rejected by their respective syngeneic hosts after transfection with B7-1, as previously described by our laboratory (Townsend and Allison, 1993). *In vitro* growth kinetics of B7SM1 was similar to that of the parental SM1 line (data not shown). B7SM1 was highly tumorigenic in syngeneic hosts. A tumor incidence of 100% was obtained when mice were

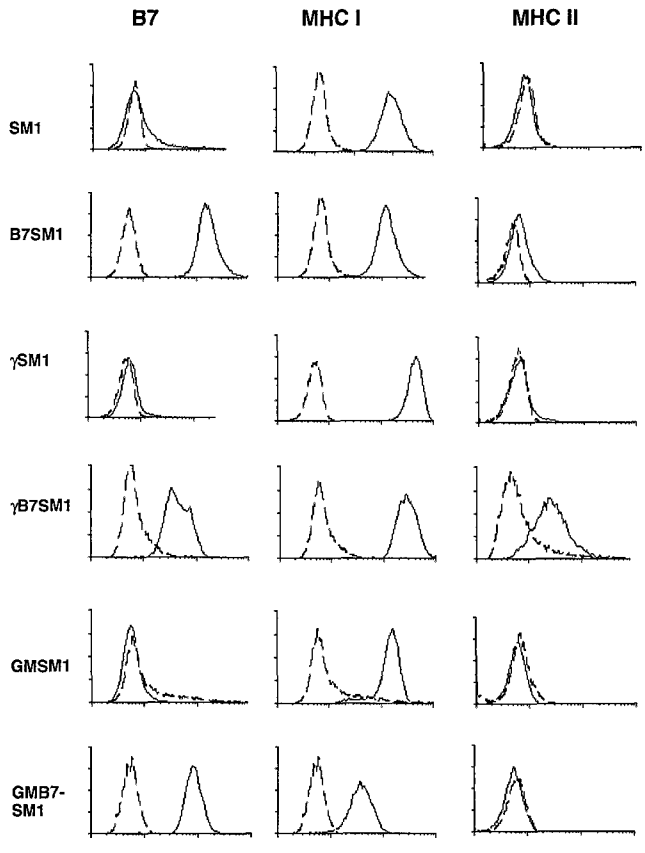

FIGURE 1 – Flow cytometric analysis of transduced SM1 cell lines. SM1 cells were lifted from tissue culture dishes by trypsinization and stained using CTLA-4 Ig or an anti-MHC antibody (solid lines) as described in Material and Methods (dashed lines represent species-matched control antibody or secondary antibody alone).

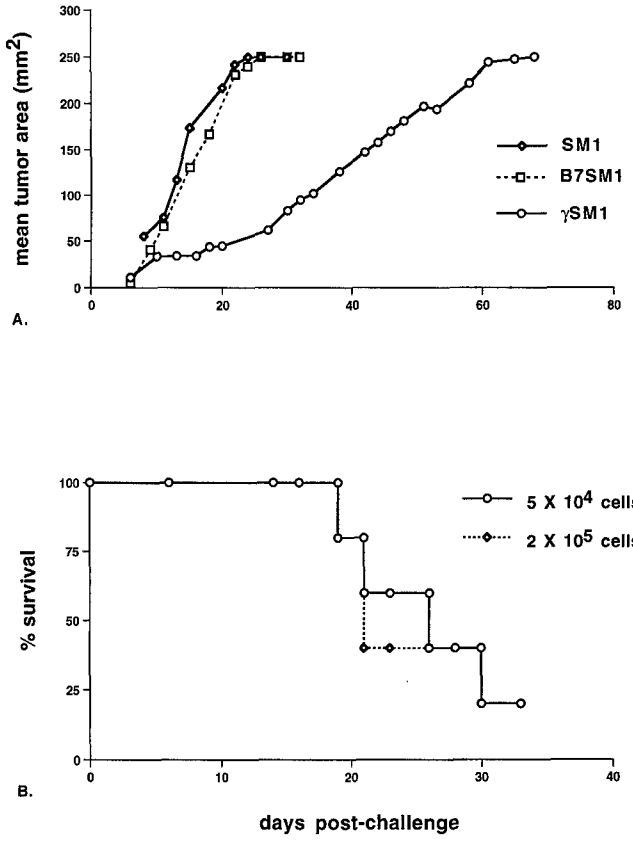

FIGURE 2 – Singly-transduced SM1 cell lines grow progressively in syngeneic mice. (*a*) Mice were challenged s.c. with 2×10^5 cells of the indicated cell line. Tumor growth was monitored by measuring bisecting diameters using a caliper. (*b*) Mice were challenged with the indicated number of GMSM1 cells and growth monitored. By 3 weeks after tumor challenge, despite only moderate tumor growth (approximately 100 mm^2), 80% of the mice began to develop a systemic illness consisting of weight loss and decreased activity. Histopathologic analysis determined that mice bearing SM1 tumors developed a myeloproliferative disorder, presumably due to GM-CSF expression by tumor cells; 100% of mice inoculated with GMSM1 eventually developed tumors.

challenged with as few as 10^4 cells. As shown in Figure 2*a*, B7SM1 grew at a rate nearly identical to the parental SM1 tumor. These results suggested that B7-1 expression by SM1 cells was not sufficient to induce rejection and that additional stimuli to boost T cell activation might be required.

IFN-γ was selected as a potent immunomodulatory cytokine that was previously demonstrated to augment anti-tumor responses (Gansbacher *et al.*, 1990; Watanabe *et al.*, 1989). SM1 cells were transduced using a retroviral vector carrying the mouse *IFN-γ* gene and cloned by limiting dilution. IFN-γ production was tested using an ELISA and a clone selected that exhibited high levels of expression (>75 ng/ml/10^6 cells/24 hr, γSM1). As shown in Figure 1, MHC class I expression was slightly higher than the parental SM1 line. This may be due to autocrine effects of the IFN-γ, as it was also observed with the γB7SM1 line.

When injected into syngeneic mice, γSM1 produced tumors that resulted in large, necrotic and ulcerated lesions within 4–5 weeks (Fig.2*a*; data not shown). Ulceration of these tumors was probably due to the pro-inflammatory effects of IFN-γ. γSM1 grew consistently slower than the parental SM1 tumor. However, like the parental SM1 and B7SM1 tumors, incidence of γSM1 was 100%. The eventual outgrowth of γSM1 was not due to cessation of IFN-γ expression because IFN-γ production by excised tumor fragments was comparable to that of γSM1 cells prior to injection (data not shown).

Since GM-CSF has previously been demonstrated to be the most effective of 10 cytokines and adhesion proteins tested to develop a vaccine for a weakly immunogenic melanoma (Dranoff *et al.*, 1993), GM-CSF was also introduced into the parental SM1 cells using retroviral transduction. Positive clones were identified by ELISA and one clone selected that expressed approximately 50 ng/ml/1 x 10^6 cells/24 hr (GMSM1). GMSM1 expressed levels of class I MHC comparable to the parental SM1 tumor and B7SM1 (Fig. 1). Growth of GMSM1 *in vitro* was similar to that of the other modified SM1 lines.

GMSM1 was injected s.c. and tumor growth monitored. GMSM1 grew progressively in BALB/c mice for approximately 2 weeks. However, when the tumors reached approximately 100 mm^2, about 80% of the mice showed signs of systemic illness (slow movement, weight loss, poor response to manual stimuli). By 3 weeks after tumor challenge, nearly half of the GMSM1-challenged mice had died (Fig. 2*b*). Upon histopathologic analysis, it was apparent that the GMSM1-challenged mice died of a myeloproliferative disease. Liver, lungs, spleen, and heart tissues all showed evidence of massive infiltration of myeloid cells which may have been a result of paracrine effects of GM-CSF produced by the live tumor inoculum. There was no definitive evidence of tumor metastasis in the organs examined. Despite not succumbing to myeloproliferative disease, after 2 to 3 weeks, the remaining mice developed tumor burdens sufficiently large enough to require euthanasia.

SM1 tumors expressing both GM-CSF and B7 are not rejected

Because expression of B7, IFN-γ, or GM-CSF alone was not sufficient to promote rejection of SM1, we tested whether boosting

both direct co-stimulation by B7 and APC recruitment/activation by GM-CSF expression would elicit rejection. B7SM1 was infected with the retrovirus containing the murine *GM-CSF* gene and a clone (GMB7SM1) selected that produced GM-CSF at levels similar to GMSM1. MHC class I expression by GMB7SM1 was slightly lower than the parental SM1 line (Fig. 1).

As shown in Figure 3*a*, implantation of GMB7SM1 resulted in tumor growth in all mice. Tumor growth rate was similar to the parental SM1 tumors at 2 doses tested (Fig. 3*a*, data not shown). Interestingly, mice challenged with GMB7SM1 did not exhibit the clinical symptoms of the myeloproliferative disorder as did the mice challenged with GMSM1.

SM1 tumors expressing both IFN-γ and B7 are rejected

We next tested whether co-expression of B7 and IFN-γ by SM1 would promote rejection. The B7SM1 line was infected with the IFN-γ-containing retrovirus and a clone (γB7SM1) was selected that produced IFN-γ at levels comparable to the γSM1 line. Similar to γSM1, γB7SM1 expressed MHC class I at levels slightly higher than the parental SM1 line. Interestingly, γB7SM1 was unique in expressing detectable levels of MHC class II. The *in vitro* growth rate of γB7SM1 was similar to that of SM1.

When injected into athymic BALB/c nude mice, γB7SM1 grew progressively at a rate similar to the parental SM1 tumor (data not shown). In contrast, γB7SM1 grew briefly in syngeneic hosts but was ultimately rejected (Fig. 3*b*). In more than 10 experiments using 2 different lines (the original γB7SM1 and a subline sorted for higher B7 expression), less than 10% (8/85) of unmanipulated mice (see below) challenged with γB7SM1 failed to ultimately reject tumors.

Blockade of IFN-γ in vivo *permits growth of γB7SM1*

The data presented above suggest that rejection of SM1 requires co-expression of both IFN-γ and B7. We tested whether IFN-γ production *in vivo* was necessary for rejection of γB7SM1. Mice challenged with γB7SM1 were treated with a neutralizing anti-IFN-γ antibody. As shown in Figure 4, administration of anti-IFN-γ resulted in growth of γB7SM1 whereas administration of (control) rat IgG had little effect on rejection. In one experiment (Fig. 4), tumors grew rapidly in all 5 anti-IFN-γ-treated mice, although one mouse eventually rejected the tumor. In a second experiment (data not shown), the dose of antibody was doubled to 1 mg/animal (i.p.) every 5 days. Again tumors grew more rapidly in the anti-IFN-γ-treated animals, although 1 of 5 mice still rejected the γB7SM1 tumor. These data confirm that expression of IFN-γ *in vivo* is required for rejection of γB7SM1.

Rejection of γB7SM1 tumors is mediated by T cells

To identify the population of T cells required for rejection of γB7SM1, mice were depleted of $CD4^+$ and/or $CD8^+$ T cell subsets prior to tumor challenge. As shown in Figure 5, γB7SM1 tumors grew in approximately 50% of mice (a total of 12/25 in 3 separate experiments) depleted of $CD4^+$ cells. In contrast, γB7SM1 grew in all mice depleted of $CD8^+$ cells, irrespective of whether the $CD4^+$ compartment was also depleted. γB7SM1 tumor growth rate in $CD8^+$ lymphocyte-depleted mice was comparable to parental tumor growth in unmanipulated mice. These data suggest that T cells mediate rejection of γB7SM1 and that the principal effector population is $CD8^+$ cells.

Rejection γB7SM1 confers immunity to rechallenge with SM1 or a related syngeneic tumor

We next examined whether mice that rejected γB7SM1 were immune to challenge with the parental tumor. Mice that rejected γB7SM1 were challenged with SM1 tumor on the contralateral flank 30 days after the initial tumor challenge. Using one γB7SM1 line, 100% protection was observed. In contrast, a second γB7SM1 tumor line (sorted by flow cytometry for high B7 expression and used in lymphocyte depletion experiments described above) conferred protection to 50% of mice (Fig. 6). Both lines expressed

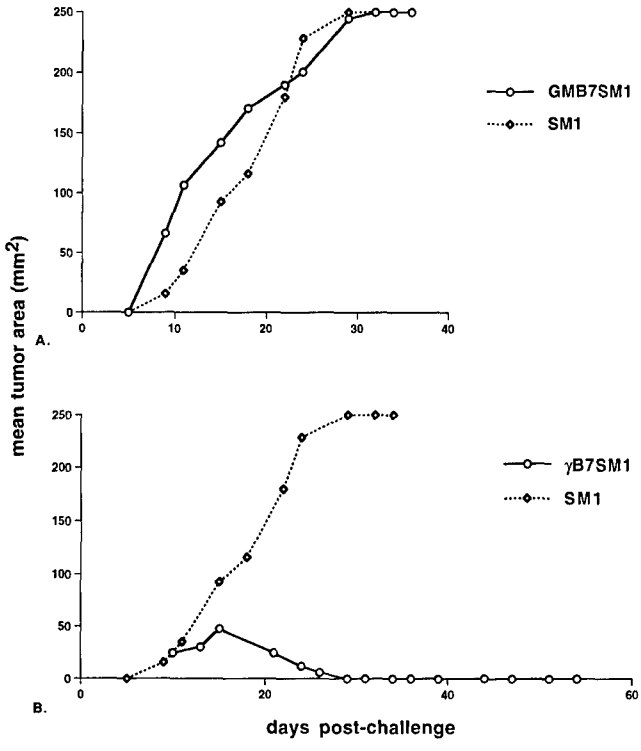

FIGURE 3 – SM1 cells co-expressing GM-CSF and B7 grow progressively whereas cells expressing IFN-γ and B7 are rejected by syngeneic mice. Mice were challenged s.c. with 2×10^5 cells of the indicated cell line and tumor growth monitored. Mice were euthanized when tumor area exceeded 250 mm².

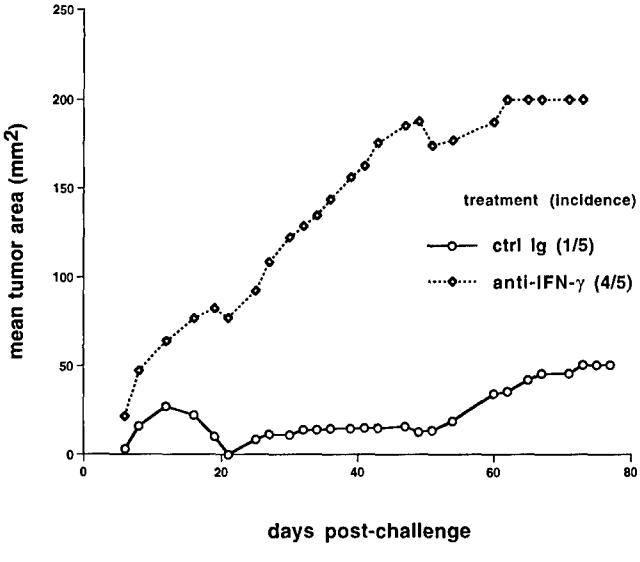

FIGURE 4 – Neutralization of IFN-γ *in vivo* abrogates rejection of γB7SM1. Mice were challenged with 2×10^5 γB7SM1 cells and treated i.p. with 1 mg of the indicated antibody on day 0. Antibody treatment was repeated every 5 days for the duration of the experiment.

comparable levels of MHC and IFN-γ. Together, these data indicate that rejection of this mammary carcinoma can result in immunity to subsequent parental tumor challenge.

To examine whether rejection of SM1 confers protection against other mammary tumors, mice that rejected γB7SM1 were rechallenged with 2 syngeneic mammary tumors. Mice that rejected

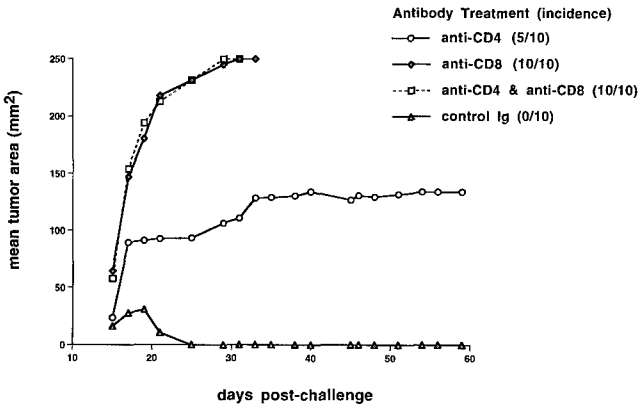

FIGURE 5 – γB7SM1 grows in mice depleted of T cell subsets. Six days prior to tumor challenge (2×10^5 γB7SM1 cells), mice were injected i.p. with the indicated antibodies as described in Material and Methods. Depletion of lymphocyte subsets was confirmed using non-cross-reacting antibodies prior to tumor challenge.

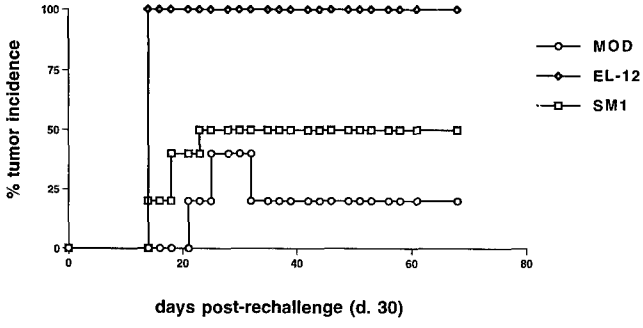

FIGURE 6 – Rejection of γB7SM1 confers protection to rechallenge with the parental SM1 tumor as well as another syngeneic mammary tumor. Mice that rejected γB7SM1 were rechallenged with the 2×10^5 cells of the indicated mammary tumor line on the contralateral flank and monitored for tumor growth. Because MOD and EL-12 exhibit significantly slower growth kinetics, data was presented as tumor incidence (tumor incidence for all 3 lines was 100% in naive mice).

γB7SM1 tumor cells were rechallenged s.c. 30 days later with either MOD or EL-12 cells. As shown in Figure 6, mice were not protected against challenge with EL-12 tumor, whereas mice were protected when rechallenged with a tumorigenic dose of the MOD tumor. These findings suggest that mammary tumors of different origins may share tumor antigens.

DISCUSSION

Many studies have demonstrated that genetic modification of tumors to express cytokine and/or co-stimulatory gene products can promote an anti-tumor immune response. However, rejection of tumors in those systems, especially B7-transduced tumors, generally correlates with the inherent immunogenicity of the tumor cells and B7 transduction by itself is not always effective. Thus, other "co-therapies" may be necessary to obtain rejection of poorly immunogenic tumors. We found that B7-1 expressing SM1 cells grew progressively in syngeneic hosts, as did SM1 variants that expressed IFN-γ or GM-CSF alone. Therefore, we tested the hypothesis that co-expression of B7 and cytokines implicated in promoting an anti-tumor response in other studies would sufficiently enhance the immunogenicity of SM1 to result in rejection of the tumor. SM1 tumors that expressed both B7 and GM-CSF grew in syngeneic hosts whereas co-expression of IFN-γ and B7 resulted in rejection of the tumor. These latter findings are consistent with another study that demonstrated that coexpression of B7-1 and IFN-γ was sufficient to promote regression of a murine neuroblastoma (Katsanis *et al.*, 1996). Lymphocyte depletion studies confirmed that T cells, especially $CD8^+$ T cells, were required for rejection of γB7SM1. Results from protection studies demonstrated that rejection of γB7SM1 provides immunity to subsequent challenge with parental SM1 tumor as well as another syngeneic mammary tumor. Taken together, these data provide evidence that tumors refractory to B7-mediated rejection possess some inherent immunogenicity that can be further augmented to promote an anti-tumor response using other immunostimulatory molecules. These findings also confirm other studies where B7 was shown to synergize with tumor-derived IL-12 expression (Coughlin *et al.*, 1995; Zitvogel *et al.*, 1996).

The lymphocyte depletion studies presented here indicate that T cells are essential in rejection of γB7SM1 tumors. Depletion of $CD8^+$ cells allowed γB7SM1 tumor growth. In contrast, approximately half of the mice depleted of $CD4^+$ cells (3 experiments, 12/25 mice) grew tumors when challenged with γB7SM1. Given the recent findings demonstrating a role of B7 in NK cell activation and tumor rejection as well as the well-established role of IFN-γ in NK cell activation (Reiter, 1993), the participation of this effector population in γB7SM1 rejection cannot be ruled out. However, our findings certainly implicate a crucial role for T cells in rejection of γB7SM1.

The demonstration that an IFN-γ-neutralizing antibody blocked rejection of γB7SM1 confirmed that IFN-γ expression *in vivo* was required for rejection. Because this depletion regimen does not distinguish between blockade of endogenous and tumor-derived IFN-γ, we cannot exclude the possibility that host-derived sources of IFN-γ may also contribute to the rejection of γB7SM1. However, these findings do confirm that upregulation of class II MHC on γB7SM1 in culture (as compared to single SM1 transfectants) was not solely responsible for its enhanced immunogenicity. Moreover, γB7SM1 tumors excised from mice subsequent to IFN-γ depletion retained class II MHC expression as determined by flow cytometric analysis (data not shown).

Administration of anti-IFN-γ blocking antibodies prevented tumor rejection, suggesting that sustained IFN-γ production was necessary for rejection. Several possible mechanisms may be responsible for the synergy between IFN-γ and B7. IFN-γ may act to simply modulate MHC expression on the tumor itself, making it more immunogenic. Alternatively, IFN-γ may serve to modulate MHC expression on host-derived APC. IFN-γ can upregulate MHC expression (class I and II) on many cell populations including dendritic cells, B cells, macrophages, and endothelial cells (Dijkmans and Billau, 1988), making them more potent APC's. Another possibility is that IFN-γ may directly activate effector cells. IFN-γ is a potent activator of both T cells and NK cells rendering them efficient cytolytic effectors (Dijkmans and Billau, 1988; Reiter, 1993). The combination of B7-mediated co-stimulation and local IFN-γ production may result in the enhanced priming of anti-tumor T cells. Finally, IFN-γ may induce expression of other immunomodulatory cytokines such as IP-10, previously demonstrated to promote T cell-mediated tumor rejection (Luster and Leder, 1993).

Further insight into this mechanism may come from the finding that unlike IFN-γ, co-expression of GM-CSF with B7 was not sufficient to promote rejection of SM1. Among the many functions of GM-CSF, perhaps the most relevant to tumor immunology is its role in APC recruitment and activation. *In vitro* studies demonstrated that exposure of bone marrow cells, umbilical cord cells, and monocytes to GM-CSF results in differentiation to dendritic cells (Inaba *et al.*, 1992). In addition, dendritic cells exposed to GM-CSF upregulate B7 and MHC class II expression, contributing to their potent APC activity (Caux *et al.*, 1994). In our study, GM-CSF expression, in combination with B7, did not promote rejection of SM1. Taken together, our findings that IFN-γ, but not GM-CSF, sufficiently enhances B7SM1 immunogenicity support

the idea that activation of the effector, but not the APC, is more effective at promoting SM1 rejection.

Vaccination using B7-expressing tumor cells principally resulted in host-derived APC presentation of tumor antigens (Huang *et al.*, 1996). Similarly, this study further showed that repeated immunization with the $B7^+$ tumors eventually resulted in priming of cytotoxic T cell effectors directly by the tumor cells. Our findings are consistent with this in that B7SM1 alone was inefficient at priming effector cells; only provision of additional activation signals resulted in tumor rejection. However, it is unclear whether the SM1 cells themselves or host APCs primed anti-tumor T cells.

Our studies also showed that enhancement of SM1 immunogenicity resulted in protection against subsequent tumor challenges. Interestingly, the 2 different sublines of γB7SM1 used resulted in different levels of protection. These lines differed in that the line that gave less protection to parental tumor rechallenge was a later-passage population sorted for higher B7 expression. This loss of protection may be due to loss of relevant tumor antigens by the clonal line that remain expressed by the parental lines. Alternatively, repeated passage of the parental line in cell culture may allow for faster growing variants to predominate in the rechallenge inoculum that evade immune surveillance based solely on high growth rate.

We also demonstrated that rejection of γB7SM1 provided protection against at least one other BALB/c-derived mammary tumor. EL-12 was derived from a pre-neoplastic mammary cell line that exhibited histological features of alveolar cells. The cell line was treated with DMBA *in vitro* and contains a point mutation in k-*ras*. MOD was also derived from a pre-neoplastic cell line that, after serial passage through mice, generated a malignant phenotype. However, MOD contains no known *ras* mutations but does have a mutation in the *p53* proto-oncogene. Both EL-12 and MOD express detectable class I MHC as determined by flow cytometry. Similar to EL-12, SM1 contains a point mutation in k-*ras* resulting in constitutive *ras* activation, although it is unclear whether this mutation alone confers tumorigenicity. Mice that rejected γB7SM1 were immune to rechallenge with the MOD tumor line but not EL-12. These findings support the idea that tumor antigens can be shared among tumors of similar tissue origins. That SM1 bears a point mutation in k-*ras*, but MOD does not, suggests that mutated *ras* may not be the relevant T cell epitope responsible for rejection of SM1.

Our findings that GM-CSF production by a dividing tumor results in systemic toxicity has important implications for immunotherapy. More than half of the mice that received a challenge with live GMSM1 tumor developed a severe myeloproliferative disorder. These findings are consistent with another study that reported toxicity of GM-CSF (Dranoff *et al.*, 1993) and suggest that vaccination with GM-CSF-expressing tumors will be best suited using irradiated tumors.

Immunotherapy of cancer has 2 goals: (1) activation of anti-tumor immunity to cure an established tumor and (2) maintenance of immunity to prevent tumor recurrence. Our present findings support the notion that tumors that do not respond to B7-based therapy can be further manipulated by cytokine-based therapy to achieve an anti-tumor immune response. Unlike protection against tumor rechallenge, successful treatment of established tumors remains elusive. Only relatively small, recently-established tumors have been eliminated using immunotherapy with singly-transduced tumors (Baskar *et al.*, 1995; Mule *et al.*, 1992). Therefore, we are addressing this aspect of immunotherapy using the transduced tumors described in this study. The synergistic effects of these regimens may focus efforts to develop a potent immunotherapeutic protocol.

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Immunology

CTLA-4 blockade synergizes with tumor-derived granulocyte–macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma

ARTHUR A. HURWITZ*, TINA F.-Y. YU, DANA R. LEACH, AND JAMES P. ALLISON

Howard Hughes Medical Institute, Cancer Research Laboratory, University of California at Berkeley, Berkeley, CA 94720

Contributed by James P. Allison, June 30, 1998

ABSTRACT **Generation of a T cell-mediated antitumor response depends on T cell receptor engagement by major histocompatibility complex/antigen as well as CD28 ligation by B7. CTLA-4 is a second B7 receptor expressed by T cells upon activation that, unlike CD28, appears to deliver an inhibitory signal to T cells. Recently, we and others demonstrated that administration of an anti-CTLA-4 antibody was sufficient to promote regression of several murine tumors. However, certain tumors, such as the SM1 mammary carcinoma, remain refractory to this type of immunotherapy. In the present study, we report that the combination of both CTLA-4 blockade and a vaccine consisting of granulocyte–macrophage colony-stimulating factor-expressing SM1 cells resulted in regression of parental SM1 tumors, despite the ineffectiveness of either treatment alone. This synergistic therapy resulted in long-lasting immunity to SM1 and depended on both $CD4^+$ and $CD8^+$ T cells. Interestingly, synergy was not observed between CTLA-4 and a B7-expressing SM1 vaccine. Given that granulocyte–macrophage colony-stimulating factor promotes differentiation and activation of dendritic cells as well as enhances cross-priming of T cells to tumor-derived antigens and that SM1 is major histocompatibility complex class II-negative, our findings suggest that CTLA-4 blockade acts at the level of a host-derived antigen-presenting cell. In addition, these results also support the idea that the most effective and synergistic vaccine strategy targets treatments that enhance T cell priming at the level of host-derived antigen-presenting cells.**

It is well established that effective T cell activation requires both an antigen-specific signal through the T cell antigen receptor and an antigen-independent costimulatory signal mediated through the interaction of CD28 with B7 on the antigen-presenting cell (APC) (as reviewed in ref. 1). Generation of an effective antitumor T cell response has these same requirements. Accordingly, the poor immunogenicity of many tumors may be because of a general lack of B7 expression. Consistent with this possibility, we and others demonstrated that conferring B7 expression to tumors of a variety of tissue origins was, in many cases, sufficient to promote tumor rejection by a $CD8^+$ T cell-dependent mechanism (2–4)

Another approach taken to enhance the antitumor immune response has been to bypass the need for direct costimulation by conferring cytokine expression to tumors. Cytokine-expressing tumor cells used as vaccines may have paracrine effects on T cells or APCs. Interleukin-2 (IL-2) (5, 6), IL-4 (7, 8), and interferon-γ (IFN-γ) (9, 10) are T cell-derived cytokines that were demonstrated to promote tumor rejection in a T cell-dependent mechanism, presumably by augmenting T cell (IL-2, IL 4, IFN-γ) or APC (IFN-γ) activation. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is another T cell-derived cytokine that was demonstrated to enhance the immunogenicity of tumors (11, 12). GM-CSF is a pleiotropic cytokine that can promote the differentiation and activation of macrophages and dendritic cells, a population of powerful APCs (13–15). In tumor model systems where neither B7 nor cytokine expression resulted in tumor rejection, it has been demonstrated that coexpression of both may be sufficient to enhance tumor immunogenicity (16, 17).

Recently, a different approach to promoting tumor rejection was described. CTLA-4 is a second T cell receptor for B7 that plays an inhibitory role in regulation of T cell responses. Several studies have demonstrated that *in vitro*, soluble anti-CTLA-4 can enhance T cell responses whereas crosslinking CTLA-4 results in blockage of cell cycle progression, diminished cytokine expression, and decreased proliferation (18–21). The observation that *CTLA-4* null mice suffer a fatal lymphoproliferative disorder supports the idea that CTLA-4 functions as a negative regulator of T cell responses. Using an antibody directed against CTLA-4, we and others demonstrated that CTLA-4 blockade enhanced rejection of B7-transfected tumors and, more strikingly, induced rejection of unmodified tumor cells and immunity to rechallenge in a T cell-dependent mechanism (22–24) (D.R.L., ●●●, unpublished data). We interpreted these data as confirming the idea that CTLA-4 delivers an inhibitory signal and that blockade of CTLA-4-mediated signals *in vivo* enhances T cell activation.

In most of the immunotherapeutic approaches studied previously, rejection of or protection against tumor challenge depended on the tumor's inherent immunogenicity. Weakly immunogenic or nonimmunogenic tumors were not rejected when genetically modified to express B7. In our studies as well, the susceptibility of tumors to CTLA-4 blockade seems to correlate with their inherent immunogenicity (D.R.L. *et al.*, unpublished data). Recently, we described a weakly immunogenic mammary carcinoma (SM1) that was not rejected when transfected with B7; SM1 tumors were rejected only when they coexpressed B7 and IFN-γ (17). These findings supported those of others and suggested that even weakly immunogenic tumors can be rejected when the immune response is enhanced sufficiently by combining immunomodulatory agents (16, 25, 26).

In the present study, we describe the rejection of SM1 tumors by using both CTLA-4 blockade and a GM-CSF-expressing tumor vaccine (GMSM1). SM1 was shown to grow progressively in mice treated with anti-CTLA-4 or the GMSM1

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Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; MHC, major histocompatibility complex; APC, antigen-presenting cell; IFN-γ, interferon-γ; GMSM1, GM-CSF-expressing SM1 tumor; B7SM1, B7-1-expressing SM1 tumor.

*To whom reprint requests should be addressed at: Cancer Research Laboratory, 415 Life Sciences Addition, University of California at Berkeley, Berkeley, CA 94720. e-mail: hurwitz@socrates.berkeley.edu.

vaccine alone. Anti-CTLA-4 treatment also was ineffective against B7–1-expressing SM1 tumors. In contrast, mice implanted with an SM1 tumor and treated with a GM-CSF-expressing vaccine followed by anti-CTLA-4 rejected the SM1 tumors and were immune to subsequent SM1 rechallenge. Not surprisingly, rejection depended on both $CD4^+$ and $CD8^+$ T cells. The finding that CTLA-4 blockade synergizes with a GM-CSF-expressing but not a B7-expressing vaccine suggests that CTLA-4 blockade may enhance tumor immunogenicity by blocking the interaction between B7 on host APCs-derived B7 and CTLA-4 on tumor-specific T cells.

MATERIALS AND METHODS

Cell Lines. The BALB/C-derived mammary carcinoma SM1 was derived in the laboratory of Satyabrata Nandi (University of California at Berkeley) (27). Briefly, a preneoplastic, BALB/C-derived mammary cell line was mutagenized with methylnitrosourea and injected into a cleared fat pad of syngeneic mice. Palpable tumors from the mammary tissue were excised and put into tissue culture after enzymatic release from extracellular matrices. Tumor lines serially passaged through syngeneic mice were selected for malignant phenotype. SM1 was selected as a line that consistently caused tumors at low inocula (tumorigenic at or below 2,000 cells) and stained positive for vimentin protein expression by immunohistochemistry. It then was cultured in MEM (University of California at San Francisco Cell Culture Facility) supplemented with 10% fetal calf serum/1× MEM nonessential amino acids/L-glutamine/MEM vitamins (BioWhittaker). Cells were lifted from tissue culture dishes by using either 5 mM EDTA in calcium/magnesium-free saline or 0.25% trypsin solution (BioWhittaker).

B7–1-expressing lines were prepared as described previously (3) by using an electroporation method. Briefly, cells in logarithmic growth phase were released from tissue culture dishes by trypsinization and washed in electroporation buffer (250 mM sucrose/1 mM magnesium chloride in 2 mM PBS, pH 7.4). DNA (100 μg) was added and 10^7 cells were electroporated by using electrodes with a 2-mm gap and a setting of 5 pulses of 99 msec at 550 V. Cells were cultured in selective medium [0.5 mg/ml G418 (Gibco)] added 48 hr after electroporation. Clones were produced by limiting dilution and screened for B7 expression by flow cytometric analysis by using CTLA-4 Ig (a chimeric molecule consisting of the extracellular domain of CTLA-4 and the Fc domain of human IgG1) followed by a fluorescein isothiocyanate-conjugated goat anti-human antibody (Caltag, South San Francisco, CA).

To obtain GM-CSF-expressing lines, cells were infected with a retrovirus containing the mouse *IFN-γ* or *GM-CSF* gene driven by the Moloney murine leukemia virus long terminal repeat, using the ψCRIP producer line (gift from Somatix, Alameda, CA). Retrovirus-containing supernatants were added to SM1 cultures overnight in the presence of 8 mg/ml polybrene (Sigma). Clones were generated by limiting dilution and supernatants were tested for cytokine expression by ELISA (PharMingen).

Animal Procedures. All animal procedures were performed according National Institutes of Health guidelines under protocols approved by the University of California Animal Care and Use Committee. SM1 cells propagated in culture were harvested with trypsin (BioWhittaker), washed three times in balanced salt solution, and resuspended in saline as described. The minimum tumorigenic dose for SM1 is 2×10^3 cells. Mice were injected s.c. into a shaved area on the back with 100 ml of tumor cell suspensions. Tumor growth was monitored by measuring bisecting diameters with a caliper. When the tumor area exceeded 250 mm^2, mice were euthanized and a value of 250 mm^2 was entered for each euthanized mouse. This value was used to calculate mean tumor area until all mice from a given group were euthanized.

In vaccination studies, cell suspensions were irradiated with 12,000 rad by using a ^{137}Cs-source irradiator. Vaccines were delivered to animals on the contralateral side from the live tumor challenge at the times indicated (generally days 0, 3, and 6).

Antibody Treatment *in Vivo*. Anti-CTLA-4 was prepared as described previously (19). Briefly, antibody-containing supernatants from the hybridoma 9H10 were bound to a Protein G-Sepharose column (Gibco) and eluted using 25 mM diethylamine. The eluate was dialyzed against isotonic saline, and antibody concentration was quantitated by UV spectrophotometry. Mice were injected with 100 μg of anti-CTLA-4 at the indicated times (generally, days 4, 7, and 10 subsequent to tumor challenge).

For lymphocyte-depletion experiments, mice were injected with anti-CD4 (GK 1.5, 400 μg), anti-CD8 (2.43, 600 μg), a combination of both anti-CD4 and anti-CD8, or control antibody (purified rat IgG, 600 μg, Sigma) three times before tumor injection (days −6, −5, and −4) as well as once every 10 days subsequent to tumor inoculation. Lymphocyte depletion was confirmed using non-cross-reactive antibodies (CD4: clone CTCD4, Caltag; CD8: CT-CD8β, Caltag) before tumor injection by testing peripheral blood or lymph node cells (from control animals) for the appropriate lymphocyte populations.

RESULTS

Transduction of SM1 with GM-CSF or IFN-γ Enhances Its Immunogenicity. We reported recently that the SM1 mammary carcinoma grows progressively, even after transduction, to express B7–1 (17). This suggested that SM1 is not strongly immunogenic. To test this directly, syngeneic mice were vaccinated s.c. with irradiated SM1 cells or the genetically modified derivative lines. Mice were rechallenged with the unmodified (parental) tumor 4–5 weeks after immunization and tumor growth was monitored (Fig. 1). In three experiments, F1 approximately half (8/15) of mice vaccinated with the parental SM1 tumor were immune to rechallenge. Consistent with our previous observations, B7 expression conferred little enhancement of immunity (9/15). In contrast, expression of IFN-γ or

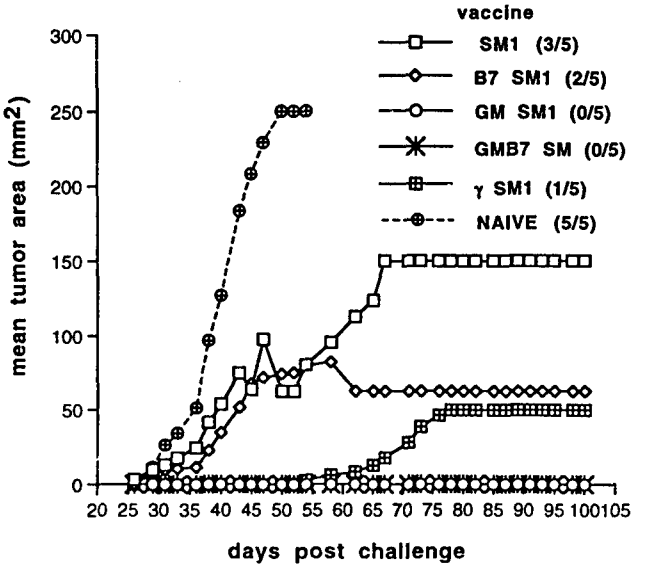

FIG. 1. SM1 is a weakly immunogenic tumor. Mice were vaccinated s.c. with 1×10^6 irradiated cells of the indicated cell line. Thirty days later, mice were rechallenged with 2×10^5 live SM1 cells and tumor growth was monitored. Incidence of SM1 tumors is indicated in parentheses.

GM-CSF significantly enhanced immunogenicity. All mice vaccinated with GMSM1, GMB7SM1, or γB7SM1 rejected a subsequent challenge with the parental SM1 tumor at an inoculum approximately 100 times the minimum tumorigenic dose. These findings were consistent with the idea that SM1 is inherently weakly immunogenic but its immunogenicity can be enhanced by transduction with genes encoding immunostimulatory cytokines such as GM-CSF or IFN-γ.

SM1 or Its B7–1-Expressing Derivative Is Not Rejected as a Consequence of Anti-CTLA-4 Treatment. We demonstrated previously that treatment with anti-CTLA-4 can enhance rejection of a $B7^+$ colorectal carcinoma (22) as well as promote the rejection of a $B7^-$ colorectal carcinoma, fibrosarcoma, and prostate carcinoma (23). To extend these findings, we tested the effectiveness of CTLA-4 blockade on the growth of SM1 and or SM1 tumors transduced to express B7–1 (B7SM1). Mice were implanted s.c. with SM1 cells and treated with anti-CTLA-4 or a control antibody 4, 7, and 10 days after tumor challenge, and tumor growth was monitored. As shown in Fig. 2, administration of anti-CTLA-4 had no significant effect on SM1 tumor growth when mice were challenged with 2×10^5 SM1 cells. Similarly, CTLA-4 blockade had no effect on B7SM1 growth when using the same-sized tumor inoculum (Fig. 2). When mice were challenged with a smaller tumor inoculum (2×10^4 cells), no significant decrease in the tumorigenicity of SM1 or B7SM1 was observed, although we did detect delayed growth of SM1 tumors (Fig. 3*a*). These findings are consistent with others from our laboratory using a variety of murine tumor models and suggest that anti-CTLA-4 treatment alone is not an effective treatment for poorly immunogenic tumors (D.R.L. *et al.*, unpublished data).

GM-CSF Expression and CTLA-4 Blockade Synergize in Treatment of SM1 Tumors. Previous studies suggested that GM-CSF is capable of enhancing antitumor immunity (11, 12, 28). As described above, GMSM1 was effective at providing immunity against rechallenge with the parental SM1 tumor. We next tested whether this vaccination strategy alone, or in combination with CTLA-4 blockade, would promote tumor regression in mice implanted with SM1 cells. Treatment with a vaccine consisting of irradiated GM-CSF-expressing SM1 cells (GMSM1) alone was not effective at promoting regression of SM1. As described above, treatment with anti-CTLA-4 resulted in delayed SM1 growth, but rarely promoted rejection (Figs. 3*a* and 4). Treatment with anti-CTLA-4 and a vaccine consisting of either SM1 or B7SM1 was not significantly more effective than anti-CTLA-4 treatment alone.

In contrast, treatment with both an irradiated GMSM1 vaccine and anti-CTLA-4 resulted in regression of the SM1 tumor in a significant fraction of animals (Fig. 3*a*). In addition, an SM1 line transduced to express both B7 and GM-CSF was equally as effective at promoting regression of SM1 tumors when used in combination with anti-CTLA-4 (Fig. 3*a*). In six separate experiments, progression of SM1 tumors after GMSM1 vaccination and anti-CTLA-4 treatment was profoundly inhibited and tumor incidence was less than 20% (7/40). We also observed regression of SM1 tumors in mice given a 10-fold-larger SM1 challenge (2×10^5 cells) and using a similar treatment protocol (5/10 mice tested, Fig. 3*b*). Together, these data suggest that CTLA-4 blockade enhances the potency of the GM-CSF-expressing vaccine.

To determine whether this treatment regimen was a result of induction of a transient effector mechanism or longer-lasting immunity, mice that rejected SM1 tumors were rechallenged with the parental tumors 30 days after regression of the initial tumor challenge. As demonstrated in Fig. 4, treatment with a GM-CSF-expressing vaccine and anti-CTLA-4 resulted in immunity to rechallenge with SM1. In two experiments, 100% of mice (10 mice) rejected rechallenge with a large dose of SM1 (2×10^5 cells). These data confirm that rejection of SM1 after GMSM1 vaccination and CTLA-4 blockade is accompanied by immunity to SM1 tumors.

Both $CD4^+$ and $CD8^+$ T Cells Are Required for Regression of SM1 Tumors. To identify the population of lymphocytes involved in rejection of SM1, mice were injected with ascitic fluid containing depleting antibodies directed against CD4 and/or CD8. After confirmation of depletion, mice were implanted with SM1 cells and treated with the GMSM1 vaccine and anti-CTLA-4 as described above. Not surprisingly, SM1 tumors grew in mice depleted of both $CD4^+$ and $CD8^+$ cells, despite a treatment regimen that was effective in mice previously administered a control rat IgG suspension (Fig. 5). Depletion of $CD8^+$ cells also resulted in tumor outgrowth, consistent with the idea $CD8^+$ cytotoxic T lymphocytes are the effector population mediating antitumor cytotoxicity. In addition, SM1 tumors also grew in mice depleted of $CD4^+$ cells alone. Given that SM1 does not express class II MHC, these data imply that GM-CSF expression by the vaccine recruits and activates host-derived APCs that present class II-restricted antigens to $CD4^+$ T cells and that this cross-priming may provide T cell help necessary for elimination of SM1 tumors. Accordingly, CTLA-4 blockade may block inhibitory interactions between these APCs and antitumor T cells.

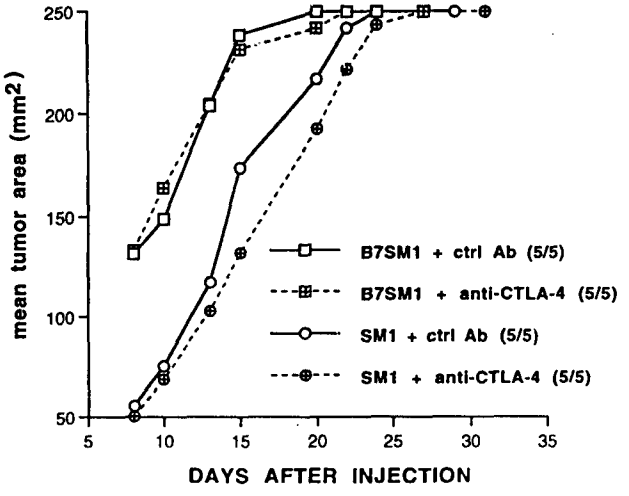

FIG. 2. SM1 is not rejected as a consequence of anti-CTLA-4 treatment. Mice were implanted s.c. with SM1 tumors (circles) or B7SM1 tumors (squares) and treated i.p. with 100 μg of either control antibody (solid lines) or anti-CTLA-4 (dashed lines) 4, 7, and 10 days later. Tumor growth was monitored, and incidence is indicated in parentheses.

DISCUSSION

We and others have shown that administration of anti-CTLA-4 can be sufficient to promote regression of unmodified tumors, presumably by blocking inhibitory signals provided by CTLA-4/B7 interactions (22–24). In the present study, we demonstrate that although CTLA-4 blockade was not effective against a weakly immunogenic mammary carcinoma, SM1, the combination of CTLA-4 blockade with a GM-CSF-expressing tumor vaccine promoted regression of SM1. This treatment strategy depended on both $CD4^+$ and $CD8^+$ T cells and induced protective immunity to rechallenge with the parental SM1 tumor.

Previously, we showed that conferring B7 expression was not effective at promoting rejection of SM1 (17). Similarly, the present study demonstrates that treatment with anti-CTLA-4 did not significantly reduce tumorigenicity of SM1, although it did slow tumor growth at lower inocula. We also demonstrated that CTLA-4 blockade does not synergize with B7 expression by SM1 cells, as administration of anti-CTLA-4 to mice implanted with B7SM1 tumors was ineffective at enhancing

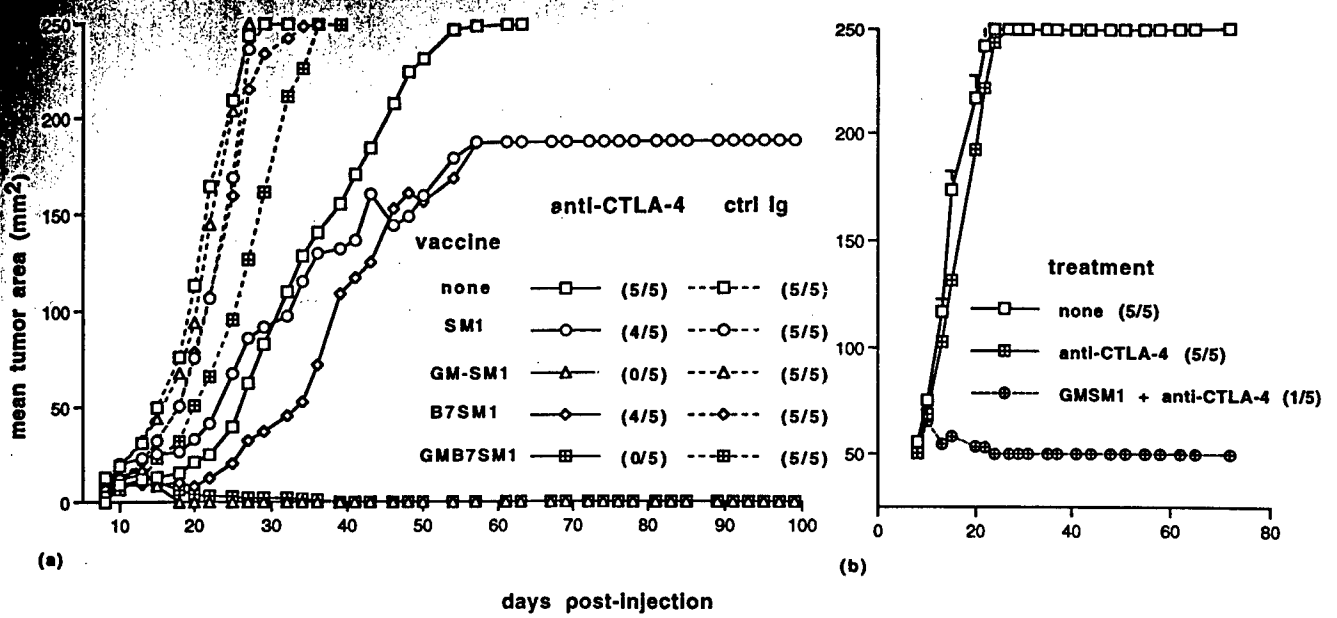

FIG. 3. GM-CSF and anti-CTLA-4 synergize in treatment of SM1 tumors. On day 0, mice were implanted with 2×10^4 (*a*) or 2×10^5 SM1 (*b*) cells. (*a*) On days 0, 3, and 6, mice were injected s.c. on the contralateral flank with the 1×10^6 irradiated cells of the indicated vaccine. On days 4, 7, and 10, mice were injected i.p. with either control antibodies (dashed lines) or anti-CTLA-4 (solid lines). (*b*) Mice were treated with a combination of anti-CTLA-4 and an irradiated GMSM1 vaccine or anti-CTLA-4 alone as described in *a*. Growth of the parental SM1 tumor was monitored, and incidence is indicated in parentheses.

rejection. Vaccination using B7SM1 and anti-CTLA-4 similarly was ineffective at promoting regression of SM1 tumors. These findings are consistent with other results from our laboratory suggesting that in poorly immunogenic tumors where B7 expression has no effect on tumor rejection, CTLA-4 blockade and B7 expression by tumors do not act synergistically to enhance antitumor immunity. These findings also may be reflective of the idea that $B7^+$ tumors may directly stimulate T cells whereas CTLA-4 blockade may act by enhancing T cell priming by host-derived APCs.

The most effective treatment strategy we observed in this study was CTLA-4 blockade in combination with a GM-CSF-expressing tumor cell vaccine. By itself, GM-CSF elicited immunity as a protective vaccine, consistent with the findings of others (11), but was ineffective at treating SM1 tumors. However, in combination with anti-CTLA-4, it was a powerful treatment for recently established SM1 tumors. Treatment of the fast-growing SM1 tumors was most effective at a dose of SM1 that is at least 10 times the minimum tumorigenic dose, suggesting that there is a threshold of treatment efficacy that is dependent on the initial tumor burden. Recently, we have extended these findings to the BL6 variant of the B16 melanoma where neither GM-CSF-expressing tumor nor anti-

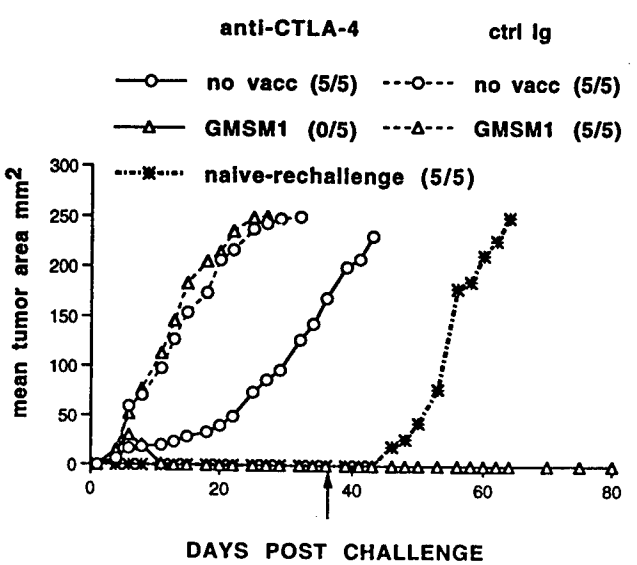

FIG. 4. Rejection as a consequence of anti-CTLA-4 treatment and a GM-CSF-expressing vaccine results in immunity to rechallenge. Mice were treated as in Fig. 3*a*. Six weeks after initial challenge with SM1, mice were rechallenged (arrow) on a separate, shaved area of the back with 2×10^5 SM1 cells. Tumor growth was monitored, and incidence is indicated in parentheses.

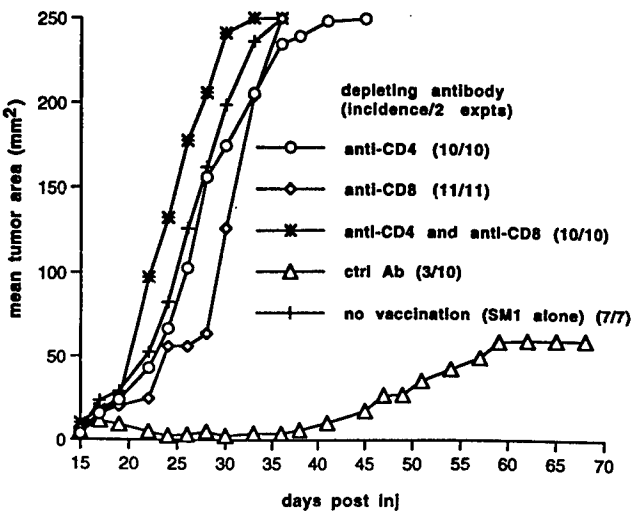

FIG. 5. Both $CD4^+$ and $CD8^+$ cells are required for regression of SM1 tumors. Six days before SM1 tumor challenge and initiation of treatment, mice were depleted of the indicated cell population as described in *Materials and Methods*. Mice were implanted with SM1 tumors and treated as described in Fig. 3*a*, and tumor growth was monitored. In contrast to mice that were mock-depleted (triangles), all mice depleted of either $CD4^+$ or $CD8^+$ cells (or both populations) grew tumors.

CTLA-4 treatment alone are effective, but the combination results in regression of tumors in about 2/3 of the mice, again with a strong dependence on initial tumor inoculum (data not shown).

Although the detailed mechanism of rejection in this system remains to be established, our studies demonstrate that both $CD4^+$ and $CD8^+$ cells are required. Because SM1 does not express MHC class II, even after exposure to IFN-γ (17), the requirement for $CD4^+$ cells suggests that class II-restricted, $CD4^+$ T cells are primed by host APCs. This idea is consistent with previous reports that tumor-derived GM-CSF enhances host presentation of tumor antigens and permits cross-priming to occur (29). GM-CSF is known to promote growth and activation of dendritic cells and render them more potent APCs (15, 30, 31). Accordingly, CTLA-4 blockade in this treatment regimen may block inhibitory interactions between host APCs (potentially GM-CSF-stimulated dendritic cells) and T cells, and facilitate costimulatory interactions between APC and T cells, thereby enhancing priming of T cells to promote immunity to and rejection of SM1.

Our previous findings suggested that both IFN-γ and B7 enhanced immunogenicity of SM1 by directly enhancing T cell activation (17). They also suggested that if "cotherapies" were to act synergistically, they both needed to activate the same "arm" of T cell activation (i.e., enhancement of T cell activation by the tumor as APC or antigen presentation by host-derived APC). Consistent with this idea, the data in the current study demonstrated that CTLA-4 blockade did not synergize with B7 expression by the tumor but it did synergize with tumor-derived GM-CSF expression. Accordingly, both tumor-derived GM-CSF expression and CTLA-4 blockade presumably enhance T cell activation at the level of host-derived APC function and therefore result in successful T cell priming. In contrast, by enhancing two different mechanisms of T cell priming (enhancing APC function of a tumor by conferring B7 expression and enhancing host APC function by CTLA-4 blockade), efficient T cell activation and, therefore, tumor clearance could not take place.

The findings presented in this report have important implications for immunotherapy in humans. Our data suggest that it is important to consider whether two therapies will act cooperatively when developing an immunotherapeutic strategy. Moreover, they also suggest that CTLA-4 blockade may be an important adjuvant for therapies in which a GM-CSF-expressing vaccine alone is inefficient. Along these lines, we currently are testing this approach to immunotherapy using two model systems of primary tumor development (27, 32). These model systems will assist in refining these and other approaches to cancer immunotherapy as well as in dissecting the mechanisms involved in T cell activation in the antitumor immune response.

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